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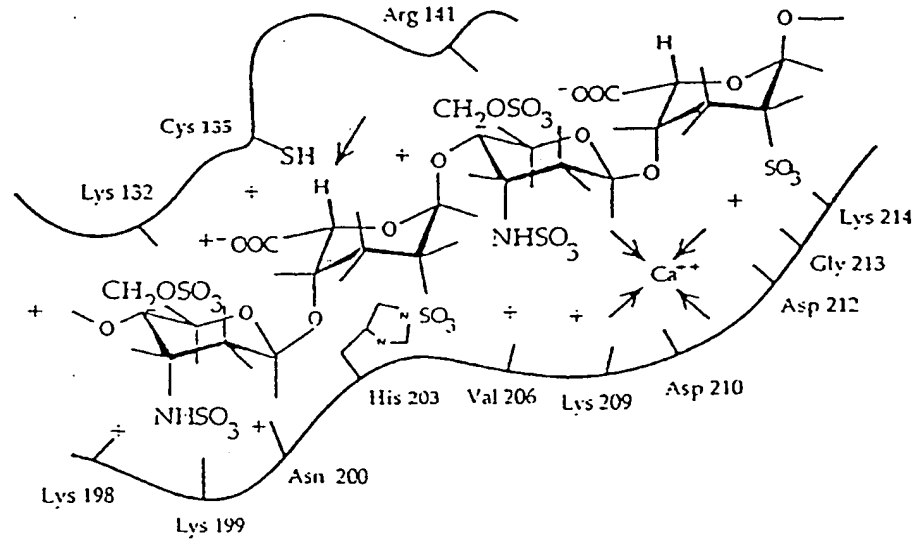

Heparin Binding and Calcium co-ordinating Sites

(57) Abstract

Modified heparinases having altered binding specificity and activity are provided. Isolated nucleic acids encoding the same as well as vectors and host cells are provided. Methods for using the modified heparinases are also provided.

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RATIONALLY DESIGNED POLYSACCHARIDE LYASES DERIVED FROM HEPARINASE I

Government Support

The present invention was supported in part by a grant from the United States National Institutes of Health (GM 25810). The U.S. government retains certain rights in the invention.

Field of the Invention

The present invention relates to polysaccharide lyases and the rational design of the same. In particular, the present invention relates to new polysaccharide lyases rationally designed and based upon the heparinase I of Flavobacterium heparinum.

Background of the Invention

The polysaccharides heparin and heparin sulfate are characterized by a disaccharide repeating unit of uronic acid and hexosamine, where the uronic acid is either L-iduronic acid or D-glucuronic acid and the glucosamine is linked to the uronic acid by a 1→4 linkage (Jackson et al., 1991). Heparin-like molecules are complex due to the high degree and varying patterns of sulfation on both the uronic acid and the hexosamine residues. It is believed that it is the sulfation which is responsible for the numerous different functional roles of these carbohydrates. Our understanding of heparin's functional role is severely limited by our poor knowledge of the heparin sequence.

Heparinases have proved to be useful tools in heparin degradation and in providing composition and sequence information (Linhardt et al., 1990). F. heparinum produces at least three types of heparinases (I, II and III) with different substrate specificities (Lohse & Linhardt, 1992). It has been proposed that all three enzymes cleave heparin through an elimination reaction catalyzed by a nucleophilic amino acid.

Heparinase I (or heparin lyase I, EC 4.2.2.7) is a 42,500 Da enzyme isolated from the periplasm of F. heparinum which cleaves heparin specifically in a random endolytic fashion (Linker and Hovingh, 1972; Linhardt et al., 1982) at linkages of the type $H_{NS,6X}$-I_{2S} or $H_{NS,6S}$-I_{2X}, where X is either sulfated or unsubstituted (Linhardt, et al., 1990; Desai, et al., 1993). The characteristic heparin degradation product profile includes $\Delta U_{2S}H_{NS}$ (disaccharide 1); $\Delta U_{2S}H_{NS,6S}$ (disaccharide 2), $\Delta U_{2S}H_{NS}I_{2S}H_{NS,6S}$ (tetrasaccharide 1), $\Delta U_{2S}H_{NS,6S}GH_{NS,6S}$ (tetrasaccharide 2), $\Delta U_{2S}H_{NS,6S}I_{2S}H_{NS,6S}$ (tetrasaccharide 3), and $\Delta U_{2S}H_{NS,6S}IH_{NAc,6S}GH_{NS,3S,6S}$ (hexasaccharide).

Heparinase I has recently been cloned and expressed in E. coli (Sasisekharan et al., 1993). The enzyme has been utilized in the sequence determination of sugars, in the preparation of small heparin fragments for therapeutic uses, and in the ex vivo removal of heparin from blood (Linhardt et al., 1990; Bernstein et al., 1988). Extracorporeal medical devices (e.g. hemodialyzer, pump-oxygenator) rely on systemic heparinization to provide blood compatibility within the device and a blood filter containing immobilized heparinase I at the effluent which is capable of neutralizing the heparin before the blood is returned to the body (Bernstein et al., 1988).

It has been suggested that heparinase I binds heparin through lysine residues on the enzyme surface (Yang et al., 1985; Linhardt et al., 1982). The importance of lysines in enzyme activity is suggested by the observation that modification by amine-reactive reagents and immobilization of heparinase I on amine-reactive supports result in extensive activity losses (Comfort et al., 1989; Leckband & Langer, 1991; Bernstein et al., 1988). Further evidence for an electrostatic nature of the interaction lies in the pH and ionic strength dependence of heparinase activity (Yang et al., 1985). Additionally, the finding that tetrasaccharides are the smallest heparin fragments that still retain substrate activity gives some information about the size requirements of the active site (Linhardt et al., 1990). Despite these observations, information concerning the structure of the enzyme has been scant.

There has been much speculation in the art about the possibility of creating "designer" enzymes, rationally designed to have desired substrate specificities and activities, and heparinase I would be an appropriate starting point for the rational design of novel polysaccharide lyases. Yet, although the importance of different levels (primary, secondary,

and tertiary) of protein structure in determining the functional activity of enzymes has long been recognized, the lack of a broad and detailed understanding of the relationship between structure and function has prevented significant progress. Even for enzymes which have known activities, substrates, and primary structures, the general lack of information about secondary and tertiary structures and the relationship of these to function has made it difficult to predict the functional effect of any particular changes to the primary structure.

Summary of the Invention

The present invention provides for new polysaccharide lyases derived from heparinase and rationally designed based upon detailed structural and functional characterization of heparinase I. In particular, in one series of embodiments, the present invention provides substantially pure polysaccharide lyases comprising the amino acid sequence of the mature heparinase I protein of F. heparinum in which at least one amino acid residue has been substituted and in which the substitution is (a) a substitution of a cysteine residue corresponding to position 135 of SEQ ID NO: 2 with a residue selected from the group consisting of aspartate, glutamate, serine, threonine, and histidine; (b) a conservative substitution of a residue of a Cardin-Weintraub-like heparin-binding sequence XBBXXXBXB corresponding to positions 197-205 or 208-212 of SEQ ID NO: 2 with a residue which conforms to the heparin-binding sequence; (c) a conservative substitution of a residue of an EF-hand-like calcium binding sequence corresponding to positions 206-220 of SEQ ID NO: 2 with a residue which conforms to the calcium binding sequence; (d) a conservative substitution of a residue of a PB1, PB2 or PB3 β-sheet domain of SEQ ID NO: 2; (e) a non-conservative substitution of a cysteine residue corresponding to position 297 of SEQ ID NO: 2; (f) a non-conservative substitution of a residue of a PB1, PB2 or PB3 β-sheet domain of SEQ ID NO: 2 which preserves a parallel ß-helix tertiary structure characteristic of SEQ ID NO: 2;(g) a deletion of one or more residues of a N-terminal region or a C-terminal region of SEQ ID NO: 2 which preserves a parallel ß-helix tertiary structure characteristic of SEQ ID NO: 2; or (h) a non-conservative substitution of a serine residue corresponding to position 39 of SEQ ID NO: 2.

The present invention thus contemplates any of the foregoing substitutions alone, but also contemplates combinations of these substitutions which result in functionally active

modified heparinases with altered stability, activity, and/or specificity as described in greater detail below.

It is, for example, a particular object of the invention to provide substantially pure polysaccharide lyases based upon heparinase I in which the cysteine residue corresponding to position 135 of heparinase I has been substituted with an aspartate, glutamate, serine, threonine, or histidine.

It is, for example, another particular object of the invention to provide substantially pure polysaccharide lyases based upon heparinase I in which the serine residue corresponding to position 39 of heparinase I has been substituted with an alanine residue.

It is, for example, another particular object of the invention to provide substantially pure polysaccharide lyases based upon heparinase I in which a residue of a Cardin-Weintraub-like heparin binding sequence XBBXXXBXB corresponding to positions 197-205 or 208-212 of heparinase I has been conservatively substituted with a residue which conforms to the heparin binding sequence. In a preferred set of embodiments, the conservative substitution is of a lysine residue corresponding to position 198, 199 or 205 of heparinase I with an arginine or histidine, most preferably an arginine. In other preferred embodiments, the conservative substitution is of the histidine residue corresponding to position 203 of heparinase I.

It is, for example, yet another particular object of the invention to provide substantially pure polysaccharide lyases based upon heparinase I in which a conservative substitution of a residue of an EF-hand-like calcium binding sequence corresponding to positions 206-220 of heparinase I with a residue which conforms to the calcium binding sequence has been made. In preferred embodiments, the substitution is of a lysine residue corresponding to position 208, 209, 211 or 214 of heparinase I with an arginine or histidine, preferably an arginine. In other preferred embodiments, the substitution is of an aspartate residue corresponding to positions 210 or 212 of heparinase I with a glutamate.

According to another aspect of the invention, there is provided a high order low molecular weight heparin fragments greater tan hexasaccharides obtainable by the process of incubating with heparin the substantially pure polysaccharide lyase of the invention (described above) to produce the low molecular weight heparin fragment. The low molecular weight heparin fragments can be separateded on an anionic exchange chromotography column (such as a POROS column from PerSeptive Biosystems).

According to another aspect of the invention, there is provided a pharmaceutical preparation comprising a sterile formulation of the substantially pure polysaccharide lyase of the invention (described above) and a pharmaceutically acceptable carrier.

According to another aspect of the invention, there is provided methods for treating subjects in need of depletion of circulating heparin. Effective amounts of the polysaccharides of the invention are administered to subjects in need of such treatment.

According to another aspect of the invention there is provided an isolated nucleic acid encoding the substantially pure polysaccharide lyase of the invention. This aspect of the invention also includes nucleic acids which hybridize under stringent hybridization conditions to the isolated nucleic acid of SEQ ID NO 1 or to the complement of the nucleic acid of SEQ ID NO 1 and which are modified to encode a modified heparinase as describedabove, and nucleic acids that differ from the nucleic acids ub codon sequence due to the degeneracy of the genetic code.

The invention further provides a recombinant host cell including any of the isolated nucleic acids of the invention.

The invention further provides an expression vector including any of the isolated nucleic acids of the invention.

According to another aspect of the invention there is provided a method of removing active heparin from a heparin containing fluid. The method involves the step of contacting a heparin containing fluid with the substantially pure polysaccharide lyase of the invention. In one embodiment of the invention the substantially pure polysaccharide lyase is immobilized on a solid support.

In another series of embodiments, the present invention provides new polysaccharide lyases in which non-conservative substitutions have been made. Thus, these embodiments include substantially pure polysaccharide lyases based upon heparinase I in which at least one amino acid residue has been substituted and in which the substitution is (a) a substitution of a cysteine residue corresponding to position 135 of heparinase I with an aspartate, glutamate, serine, threonine or histidine residue; (b) a substitution of a histidine residue corresponding to

position 203 of heparinase I with an aspartate, glutamate, serine, threonine or cysteine; (c) a substitution of a lysine residue corresponding to position 198, 199, 205, 208, 209, 211 or 214 of heparinase I with a small non-polar amino acid, a small polar amino acid, or an acidic amino acid; (d) a substitution of a small polar or small non-polar amino acid for a residue corresponding to the Phe197, Asn200, Asp204, Glu207, Asp210, Asp212 or Gly213 of heparinase I.

In this series of embodiments, the present invention provides for single substitutions and also provides for polysaccharide lyases with combinations of these substitutions as discussed above.

In another series of embodiments, the present invention provides for a modified heparinase having a modified heparinase k_{cat} value, wherein the modified heparinase k_{cat} value is ≤75% of a native heparinase k_{cat} value of a complementary native heparinase. The complementary native heparinase for modified heparinase I is, of course, heparinase I.

In another series of embodiments the modified heparinase is immobilized on a solid support membrane.

In another series of embodiments, the present invention also provides for polysaccharide lyases in which the overnight heparin degradation activity is less than about 75% of that of native heparinase I.

In another series of embodiments, the present invention also provides for polysaccharide lyases in which the degradation product profile is altered from that of native heparinase I. In one embodiment the polysaccharide lyase is a modified heparinase I having a modified product profile, wherein the modified product profile of the modified heparinase I is ≤50% similar to a native product profile of a native heparinase I. In another embodiment the substantially pure polysaccharide lyase is a modified heparinase I producing when contacted with heparin less than 20% of disaccharide 1 and trisaccharides 2 and 3 as compared to native heparinase I when contacted with the heparin.

In another series of embodiments, the present invention also provides active fragments and functionally equivalent variants thereof of the polysaccharide lyases of the invention that have substantially the same heparinase I activity as the substituted polysaccharide lyases of the invention.

Brief Description of the Drawings

Figure 1 presents a "side" view of the parallel β-sheet structure of the core of heparinase I in which Cys135 is marked by an arrow.

Figure 2 presents a view "down" the axis of the parallel β-helix of the core of heparinase I in which Cys135 is marked by an arrow.

Figure 3 shows a heparin tetrasaccharide bound in the heparin binding domain. Cys135 and His203 are in close proximity to each other and the hydrogen of the iduronate marked by an arrow.

Detailed Description of the Invention

The present invention provides a series of new polysaccharide lyases derived from the heparinase I (heparin lyase I, EC 4.2.2.7) of F. heparinum. In particular, based upon a detailed structural and functional characterization of heparinase I, new polysaccharide lyases with altered stability, activity and specificity are provided.

The nucleotide and amino acid sequences of heparinase I are provided in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. These sequences were reported in Sasisekharan, et al. (1993) and provided the first insight into the primary structure of the native heparinase I of F. heparinum.

The present disclosure provides a wealth of additional information about the secondary and tertiary structure of this polysaccharide lyase as well as information relating to the functional roles of the various regions of the enzyme. This information is based upon detailed biochemical mapping of the active site and polysaccharide binding domain, characterization of these sites through kinetic studies, characterization of mutants created by site-directed mutagenesis, and computer-based modeling of secondary and tertiary structures. The result is a detailed picture of the primary, secondary, and tertiary structures of heparinase I and the functional roles of various regions of the enzyme.

Using this detailed knowledge of the native heparinase I of F. heparinum, the present disclosure provides novel, rationally designed polysaccharide lyases, methods of designing and producing such lyases, and uses therefor.

Heparinase I has a typical prokaryotic leader sequence and cleavage site. This leader sequence corresponds to residues 1-21 of SEQ ID NO: 2. A recombinant construct lacking the leader (L) residues, designated -L r-heparinase I, has been expressed in a pET plasmid and the recombinant enzyme is still as active as the native F. heparinum heparinase, indicating that these residues are not essential for enzymatic activity. Inclusion of the leader in the mature protein, however, does not interfere with the enzyme's activity (Sasisekharan et al., 1993).

Although the full heparinase I amino acid sequence includes three cysteines, one of these (at position 17 of SEQ ID NO: 2) is in the leader sequence. Therefore, the mature heparinase I has two cysteines. These cysteines are at positions 135 ("Cys135") and 297 ("Cys297") of SEQ ID NO: 2.

Previous studies had suggested that the two cysteine residues of mature heparinase I form a disulfide bridge. Comfort et al. (1989), for example, compared the activity of heparinase I in which the cysteine residues had been reduced with heparinase in which the cysteines had not been altered. These experiments showed that the reduced heparinase I had lower activity than the native heparinase I. Therefore, it was suggested that the two cysteine residues may form a disulfide bridge in native heparinase I and that the disulfide bridge may be important to maintaining tertiary structure and activity.

Studies using sulfhydryl modifications, kinetics of enzyme inactivation, and competitive inhibition of inactivation were performed by the present inventors to determine the functional roles of the two cysteines in catalysis. Purified heparinase I preparations (Example 1) were modified with various sulfhydryl specific reagents to map and characterize the cysteine residues.

We now disclose (a) that the two cysteines of heparinase I do not form a disulfide bridge but, rather, are located in different parts of the tertiary structure with different microenvironments, (b) that Cys135 is surface-accessible, (c) that Cys135 is in the active site, (d) that the microenvironment around Cys135 is positively charged, and (e) that Cys135 is involved

in catalysis. In contrast, Cys297 is buried in the hydrophobic core of the protein and is not essential to heparinase I activity. See Examples 2-8.

To further demonstrate that Cys135 is an active site cysteine involved in catalysis and that Cys297 is not essential to enzyme activity, recombinant polysaccharide lyases were produced in which these residues were modified. Using site-directed mutagenesis, Cys135 was replaced by the more weakly nucleophilic residue serine, by the charged residues histidine, glutamate and aspartate, and by the neutral alanine. Replacement of Cys135 by aspartate, glutamate, serine or histidine led to decreases in activity (k_{cat} values of approximately 3.5%, 3.8%, 2% and 3%, respectively, of the k_{cat} of native heparinase) and replacement with the neutral alanine abolished activity. Importantly, the cleavage specificity of the recombinant lyases was unaffected. Replacement of Cys297 with either serine or alanine had no effect on activity. See Example 9.

Next, we investigated the heparin-binding domain of heparinase I using activity analysis at varying calcium concentrations, heparin affinity chromatography, affinity co-electrophoresis, heparin blotting of CnBr digests, competitive binding and blotting of tryptic digests, competition with a synthetic binding domain peptide, PCMB protection and tryptic digests, and site-directed mutagenesis of the binding domain. See Examples 10-17.

We now disclose that (a) heparinase I possesses a lysine-rich heparin binding domain extending approximately from residues 195-220 of SEQ ID NO.:2, (b) that the binding domain possesses two "Cardin-Weintraub" heparin binding sequences at approximately residues 197-205 and 206-212, (c) that heparinase activity is calcium dependent and the heparin binding domain contains an EF-hand-like calcium binding site at approximately residues 206-220, and (d) that the heparin binding domain is in close proximity to Cys135 in native heparinase I.

As noted above, the heparin binding domain possesses two sequences which almost conform to consensus sequences found in many heparin binding proteins (Cardin and Weintraub, 1989). The Cardin-Weintraub sequence is of the form XBBBXXBX or XBBXBX (where B is any basic residue and X is any hydrophobic or other residue). The heparinase I sequences which nearly conform to these sequences are found at positions 197-205 and 206-212 of SEQ ID NO.:2. Using site-directed mutagenesis, recombinant proteins were produced in which these sites were

altered. A third Cardin-Weintraub sequence, at approximately positions 331-337, does not appear to be involved in heparin binding.

Also within the heparin binding domain is a sequence which nearly conforms to an EF-hand calcium binding domain (Kretsinger et al., 1991) at positions 206-220. The EF-hand consensus sequence is shown in Table II. Substitutions conforming to an EF-hand calcium binding consensus sequence, but which would not conform to a Cardin-Weintraub heparin binding sequence, appear to be tolerated but a deletion of the entire sequence leads to enzyme inactivity. For example, substitution of both Lys208 and Lys209 with the similarly positive arginine led to a 40% decrease in initial activity (k_{cat}) but had no effect on the product profile. This substitution is consistent with both the EF-hand and Cardin-Weintraub consensus sequences. Substitution of both of these residues with the neutral alanine did not significantly alter the product profile but led to a 76% decrease in k_{cat}. This substitution conforms to the EF-hand consensus but not the Cardin-Weintraub consensus. Moreover, substitution of both lysines with negative aspartate residues results in a decrease in k_{cat} of only 46% and an unaltered product profile, further showing that the amines of these lysine residues are not necessary for catalysis. Therefore this stretch of the sequence is primarily a calcium binding site and, secondarily, a heparin binding sequence. Deletion of this region abolishes activity, suggesting either that calcium is necessary to activity or that the deletion disrupts the tertiary structure of the active site. A second EF-hand domain, at approximately positions 372-384, does not appear to be involved in catalysis.

With respect to the heparin binding site at residues 197-205, a different picture emerges. This sequence nearly conforms to a Cardin-Weintraub heparin binding sequence, having the motif XBBXXXBXB. This sequence does not conform to an EF-hand calcium binding site. Substitution of positively charged arginines for lysines (Lys198 and Lys199) conserves the Cardin-Weintraub-like motif and results in a 46% decrease in k_{cat} but no change in product profile. Interestingly, substitution of Lys198 and Lys199 with either the neutral alanine or negatively charged aspartate does not abolish activity but, rather, results in lower activity (k_{cat} 4.1% of wild type) and an altered product profile. Another basic residue in this domain, His203, appears to be involved in the active site as an acid/base catalyst. Substitution of His203 with

nucleophilic negative or polar residues which do not conform to the Cardin-Weintraub sequence (e.g., aspartate, serine or cysteine) does not inactivate the enzyme but, rather, results in a lyase with decreased activity (k_{cat} 3-4% of wild type). Substitution with the neutral alanine, however, abolishes activity, suggesting that this residue may be important in proton transfer.

Using the information relating to the active site and heparin binding domain of heparinase I, in conjunction with computer database searches for homologous sequences of known structure, computer programs for the prediction of secondary structures, and computer-assisted modeling, we have developed a model of the tertiary structure of native heparinase I.

We now disclose that heparinase I is characterized by a parallel β-helix structure. A similar structure has recently been reported for two pectate lyases (Yoder et al., 1993). For much of this structure, each turn of the helix consists of two or three β-strands separated by non-β stretches. The turns of the helix are stacked such that the β-strands of adjacent turns may form parallel β-sheets. Thus, the helix is three-sided for most of the core structure with the three sides consisting largely of three parallel β-sheets. The three parallel β-sheets forming the sides of the helical core are designated PB1, PB2, PB3. In each turn, the β-strands are between two and seven residues in length and each turn includes a minimum of seventeen residues and an average of about nine residues in β-strands. Between β-strands are loops of varying lengths. Both Cys135 and the heparin binding domain are found in such loops. Table I discloses the approximate positions of the residues of heparinase I which are found in the β-strands and to which of the β-sheets each β-strand belongs. Schematic views of the scaffold of Table I, omitting some of loops between β-strands, are presented in Figures 1 and 2. Figure 1 presents a "side" view in which Cys135 is marked by an arrow. Figure 2 presents a view "down" the axis of the parallel β-helix and, again, Cys135 is marked by an arrow.

Note that the active site cysteine, Cys135, is located in a non-β-stretch between PB1 and PB2. This is consistent with the results of the sulfhydryl modification experiments which indicated that Cys135 is surface accessible. Cys297, on the other hand, is part of a β-strand in PB1 and its side chain is believed to be directed toward the hydrophobic interior of the helix. The heparin binding domain loops out of a turn between PB3 and PB1 and extends back up the β-helix toward Cys135. This structure places the active site cysteine in proximity to the heparin

binding domain and explains the positively charged microenvironment of Cys135 and the inhibition of the PCMB reaction with Cys135 by the addition of heparin.

From the results disclosed herein, a molecular mechanism for the interplay between heparin binding and catalysis by heparinase I emerges. Cys135 is catalytically active, but is not a determinant for heparin binding, since chemically derivatizing it did not affect heparinase binding to heparin. It is proposed that this residue abstracts the C5 proton on the uronate of the disaccharide repeat unit of the acidic polysaccharides, and initiates the elimination based depolymerization reaction. The thiol group of cysteine has a pK_a of 8.35 in free solution (Fresht, 1985), indicating that this residue will be fully protonated at pH 7.0, the pH optimum for heparinase I (Yang et al., 1985). A positively charged environment from nearby lysines or arginines, however, will tend to keep the thiol group negatively charged (i.e. lowering its pK_a) so that it can act as a base for proton abstraction. This would account for the preferential reactivity of Cys135 with negatively charged reagents and the high reactivity of Cys135 at pH 6.5 (Example 6).

The heparin binding domain (residues 195-220) controls enzymatic selectivity in terms of substrate size, and the histidine from this region (His203) also assists the catalytic mechanism, possibly by acting as a secondary nucleophile or as an essential amino acid in a possible "proton relay system." This site also contains the calcium co-ordination site which bridges heparin to heparinase through calcium, and perhaps orients the functional group(s) of the uronate to the active site region involving Cys135 and His203. The heparin binding site and the basic residues close to Cys135 (e.g., Lys132, Arg141, etc.) together constitute a heparin binding domain in heparinase I that provide the basic environment for Cys135. Substitution of lLysine132 to an alanine residue reduced the activty of the haprinase (see table IV). This positive charge environment around the surface accessible Cys135 enhances the reactivity of this thiol residue. Thus, we postulate that a positively charged heparin binding domain spatially close to Cys135 provides the necessary charge complementarity for very specific heparin binding on the one hand, while on the other it provides for the active site environment which plays a key role in biasing the active site reactivity. Figure 3 shows a schematic representation of the active site of heparinase I with a heparin tetrasaccharide bound in the heparin binding domain. Cys135 and

His203 are in close proximity to each other and to the hydrogen of the iduronate marked by an arrow.

In light of the present disclosure, one of ordinary skill in the art is now able to rationally design new polysaccharide lyases with altered activity and specificity. In particular, one is able to design lyases with altered activity by the modification of Cys135 or the positively charged residues surrounding it in the active site or the heparin binding domain. In addition, one is enabled to design modified polysaccharide lyases with altered specificity by modification of the residues of the Cardin-Weintraub-like sequence in the heparin binding domain. Finally, one is able to produce various other novel polysaccharide lyases in which non-essential residues are freely changed or substituted conservatively.

Preferred Embodiments

The present invention provides for novel polysaccharide lyases rationally designed on the basis of the sequence of the heparinase I of F. heparinum and the structural and functional characterizations disclosed herein.

In the description that follows, reference will be made to the amino acid residues and residue positions of native heparinase I disclosed in SEQ ID NO: 2. In particular, residues and residue positions will be referred to as "corresponding to" a particular residue or residue position of heparinase I. As will be obvious to one of ordinary skill in the art, these positions are relative and, therefore, insertions or deletions of one or more residues would have the effect of altering the numbering of downstream residues. In particular, N-terminal insertions or deletions (e.g., deletion of one or more of the 21 N-terminal leader sequence residues) would alter the numbering of all subsequent residues. Therefore, as used herein, a residue in a recombinant polysaccharide lyase will be referred to as "corresponding to" a residue of the full heparinase I if, using standard sequence comparison programs, they would be aligned. Many such sequence alignment programs are now available to one of ordinary skill in the art and their use in sequence comparisons has become standard (e.g., "LALIGN" available via the internet at http://genome.eerie.fr/fasta/). As used herein, this convention of referring to the positions of residues of the recombinant polysaccharide lyases by their corresponding heparinase I residues

shall extend not only to embodiments including N-terminal insertions or deletions but also to internal insertions or deletions (e.g, insertions or deletions in "loop" regions).

In addition, in the description which follows, certain substitutions of one amino acid residue for another in a recombinant polysaccharide lyase will be referred to as "conservative substitutions." As used herein, a "conservative amino acid substitution" or "conservative substitution" refers to an amino acid substitution in which the substituted amino acid residue is of similar charge as the replaced residue and is of similar or smaller size than the replaced residue. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) the small non-polar amino acids, A, M, I, L, and V; (b) the small polar amino acids, G, S, T and C; (c) the amido amino acids, Q and N; (d) the aromatic amino acids, F, Y and W; (e) the basic amino acids, K, R and H; and (f) the acidic amino acids, E and D. Substitutions which are charge neutral and which replace a residue with a smaller residue may also be considered "conservative substitutions" even if the residues are in different groups (e.g., replacement of phenylalanine with the smaller isoleucine).

Additionally, some of the amino acid substitutions are non-conservative substitutions. In certain embodiments where the substitution is remote from the active or binding sites, the non-conservative substitutions are easily tolerated provided that they preserve the parallel B-helix tertiary structure characteristic of native heparinase (SEQ ID NO: 2), thereby preserving the active and binding sites.

As shown in the experimental examples below and, in particular Table IV, the present invention provides a variety of novel polysaccharide lyases with heparin-cleaving activity in which the initial activity or k_{cat} is reduced relative to the k_{cat} of native heparinase I. For each of the novel polysaccharide lyases tested thus far, the k_{cat} of the recombinant lyase is less than about 75% of that of native heparinase I. Such lyases have particular utility as substitutes for heparinase I in the controlled degradation of heparin and other polysaccharides because the reaction proceeds at a slower rate. Table IV also shows the percentage of degradation products, relative to native heparinase I, produced by these new lyases after overnight incubation with heparin. In all cases but two, discussed below, the product profile of these new lyases is essentially identical to the characteristic heparinase I product profile.

The novel polysaccharide lyases of the invention are rationally designed based upon the sequence of heparinase I and have at least one amino acid substitution at the active site Cys135, in the first Cardin-Weintraub-like sequence in the heparin binding domain, or in the EF-hand-like calcium binding sequence in the heparin binding domain. With respect to Cys135, the substitutions are preferably substitutions of nucleophilic or negatively charged residues which can replace the functional role of Cys135 in catalysis. With respect to the Cardin-Weintraub-like and EF-hand-like sequences, the substitutions are preferably conservative substitutions which preserve the motif of the sequence but, as shown in the examples below, these substitutions need not be conservative in order to maintain enzymatic activity.

Thus, as a first preferred embodiment, the present invention provides a novel polysaccharide lyase comprising the mature peptide amino acid sequence of SEQ ID NO: 2 (i.e. residues 22-384) in which the residue corresponding to Cys135 has been substituted by a serine, histidine, glutamate or aspartate residue. Recombinant lyases with such a substitution have been found to have reduced initial activity (k_{cat}) and have particular utility when partial or slower heparin degradation is desired. Such recombinant polysaccharide lyases may optionally include the heparinase leader sequence or may include any of the other modifications described herein.

In another set of embodiments, the present invention provides novel polysaccharide lyases comprising the mature peptide amino acid sequence of SEQ ID NO: 2 in which one or more of the residues of the Cardin-Weintraub-like sequence, XBBXXXBXB, corresponding to positions 197-205 are substituted. For example, one or more of the lysine residues corresponding to positions 198, 199 or 205 of the heparin binding domain may be substituted by other positively charged residues. Such substitutions maintain the positively charged microenvironment necessary for heparin binding and catalysis by Cys135. These substitutions may be conservative, such as replacing one or more of the two lysine residues (corresponding to positions 198 and 199 or SEQ ID NO: 2) with an arginine or histidine. Replacement of both of these residues with arginine leads to the creation of a lyase with 60% the k_{cat} of native heparinase I. Alternatively, one or more of these lysines may be substituted with a small polar, small non-polar or even an acidic amino acid residue to produce a lyase with reduced activity. For example, recombinant lyases with both of these lysines replaced by alanine or aspartate have been shown to retain

catalytic activity but have k_{cat} values only about 4% of the native heparinase I. The lysine corresponding to position 205 of SEQ ID NO: 2, also may be modified. Substitution with an arginine or histidine is one preferred embodiment. Alternatively, this residue may be substituted with a small polar, small non-polar or acidic amino acid residue. Substitution of one of these residues with a much larger residue (e.g. tyrosine), however, abolishes activity. As noted above, the histidine at position 203 of heparinase I appears to be involved in the active site as an acid/base catalyst. Replacement of this residue by a neutral residue (e.g. alanine) abolishes catalytic activity. This residue may, however, be substituted, by serine, cysteine, threonine, or even the negative aspartate or glutamate to produce a polysaccharide lyase with a reduced k_{cat}.

The non-basic residues in the region corresponding to this first Cardin-Weintraub-like sequence may also be modified while preserving the XBBXXXBXB motif by making conservative substitutions. In order to maintain heparin binding ability, the substitutions are preferably conservative with respect to residue size (e.g. Phe197 → Tyr; Ile201 → Leu) and charge (e.g., Ala202 → Gly; Asp204 → Glu). Alternatively, small polar or small non-polar residues may be substituted for any of these residues. The residue corresponding to Asn200 in SEQ ID NO: 2, for example, may be changed to a glutamine to produce a recombinant lyase. Substitution of Asn200 with alanine results in a recombinant lyase with a k_{cat} of approximately 48% wild-type. The substitution of this asparagine with a lysine residue, however, is not tolerated because it is not a conservative substitution with respect to size (i.e. lysine is larger than asparagine).

In another set of embodiments, novel polysaccharide lyases are provided comprising the mature peptide amino acid sequence of SEQ ID NO: 2 wherein one or more residues corresponding to the EF-hand-like calcium binding domain (residues 206-220) have been substituted but calcium binding ability and catalytic activity is retained. Preferably, these substitutions are conservative with respect to size and charge. Thus, one or more of the lysines corresponding to positions 208, 209, 211 or 214 may be substituted with arginine or histidine. Alternatively, one or more of these residues may be substituted with a small polar or small non-polar residue. For example, replacement of Lys214, which is not constrained in the EF-hand motif, with Ala resulted in a lyase with 60% of the k_{cat} of the wild type. The aspartate residues corresponding to positions 210 and 212 of SEQ ID NO: 2 are constrained in the EF-hand

sequence motif. Replacement of these residues by glutamate, however, would conform with the motif and would also constitute a conservative substitution with respect to charge and approximate size. Conservative substitutions of the neutral residues of this region may also be made as well as substitutions with small polar or small non-polar residues.

The EF-hand-like calcium binding domain has also been shown to be tolerant to some substitutions which do not conform to the EF-hand motif. Thus, for example, the substitutions of Glu207 → Ala, Asp210 → Ala, Asp212 →Ala, and Gly213 → Ala do not destroy catalytic activity but result in lyases with k_{cat} values of 18%, 50%, 50%, and 20%, respectively, of the k_{cat} of native heparinase I. As with the lyases with modified heparin binding domains, these recombinant lyases with reduced k_{cat} have utility in that they allow for slower and more controlled degradation of heparin and other polysaccharides. In some cases double and triple mutations in the EF-hand region produce novel polysaccharide lyases having even lower enzymatic activity.

In another embodiment a novel polysaccharide lyase is provided which retains all of the enzymatic activity of the heparinase I but which is not immunogenic when administered to a subject. This novel polysaccharide lyase is produced by making a non-conservative substitution of the serine at position 39 of heparinase I which removes the gycosylation site of heparinase I. The novel substituted polysaccharide lyase is not immunogenic because the immunogenic region (glycosylation) of the lyase is removed. Preferably the serine residue corresponding to position 39 of heparinase I is substituted with an alanine residue.

In one particular embodiment, the present invention provides novel polysaccharide lyases in which the product profile is different from that of native heparinase I. In particular, polysaccharide lyases comprising the mature peptide amino acid sequence of SEQ ID NO: 2 in which the residues corresponding to Lys198 and Lys199 have been replaced by negatively charged residues (i.e. Asp or Glu) produce less than 20% and, in fact, only negligible amounts of the characteristic disaccharide 1, tetrasaccharide 2 and tetrasaccharide 3 of the heparinase I product profile. Relative to heparinase I, these recombinant lyases, after overnight incubation with heparin, produce about 50% of the characteristic disaccharide 2 and tetrasaccharide 1. These lyases have particular utility in the sequencing of heparin and other complex polysaccharides.

The product profile produced by the novel polysaccharide lyases includes high order low moleuclar weight heparin fragments which are not included in the product profile produced by native heparinase I. The high order low molecular weight fragments are large undigested fragments of heparin which have various levels of therapeutic activity attributed to larger fragments of heparin.

In another set of embodiments, the invention provides novel polysaccharide lyases comprising the mature peptide amino acid sequence of SEQ ID NO: 2 in which one or more residues outside of the active site and heparin binding domain have been substituted so as to preserve the overall tertiary structure of the enzyme. In particular, polysaccharide lyases in which conservative substitutions of any of the residues of the β-strands of Table I are contemplated. Because these residues are now known not to be involved in catalysis, and because a basic model of the tertiary structure of heparinase I is now disclosed, such conservative substitutions may be made without undue experimentation and with a high expectation of success. In a particularly preferred embodiment, the residue corresponding to Cys297 may be substituted by a small polar or non-polar residue (e.g., Cys297 → Ser or Cys297 → Ala) without affecting enzyme activity. Although the Cys297 residue has now been shown to be irrelevant to protein activity, modification of this residue is particularly contemplated in preferred embodiments to increase stability and simplify mass production and purification by removing the possibility of unwanted disulfide cross-linking with Cys135.

The substantially pure polysaccharide lyase of the invention may also be used to remove active heparin from a heparin containing fluid. A heparin containing fluid is contacted with the substantially pure polysaccharide lyase of the invention to degrade the heparin. The method is particularly useful for the ex vivo removal of heparin from blood. In one embodiment of the invention the substantially pure polysaccharide lyase is immobilized on a solid support as is conventional in the art. The solid support containing the immobilized polysaccharide lyase may be used in extracorporeal medical devices (e.g. hemodialyzer, pump-oxygenator) in which systemic heparinization to prevent the blood in the devise from clotting. The support membrane containing immobilized heparinase I is positioned at the end of the devise to neutralize the heparin before the blood is returned to the body.

According to another aspect of the invention, there is provided methods for treating subjects in need of depletion of circulating heparin. Effective amounts of the polysaccharides of the invention are administered to subjects in need of such treatment. For example, subjects undergoing open heart surgery or hemodialysis often are in need of depletion of medically undesirable amounts of heparin in blood as a result of blood as a result of the surgery or hemodialysis. The subjects may be administered the modified heparinases of the invention in a manner and in amounts presently found acceptable when using native heparin. Effective amounts are those amounts which will result in a desired reduction in circulating heparin levels without causing any other medically unacceptable side effects. Such amounts can be determined with no more than routine experimentation. It is believed that doses ranging from 1 nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, will be effective. The absolute amount will depend upon a variety of factors (including whether the administration is in conjunction with other methods of treatment, the number of doses and individual patient parameters including age, physical condition, size and weight) and can be determined with routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. The mode of administration may be any medically acceptable mode including oral, subcutaneous, intravenous, etc.

One of ordinary skill in the art, in light of the present disclosure, is now enabled to produce substantially pure preparations of any of these novel polysaccharide lyases by standard recombinant technology. That is, one may substitute appropriate codons in SEQ ID NO: 1 to produce the desired amino acid substitutions by standard site-directed mutagenesis techniques. Obviously, one may also use any sequence which differs from SEQ ID NO: 1 only due to the degeneracy of the genetic code as the starting point for site directed mutagenesis. The mutated nucleic acid sequence may then be ligated into an appropriate expression vector and expressed in a host such as F. heparinum or E. coli. The resultant polysaccharide lyase may then be purified by techniques well known in the art, including those disclosed below and in Sasisekharan, et al. (1993). As used herein, the term "substantially pure" means that the proteins are essentially free of other substances to an extent practical and appropriate for their intended use. In particular, the proteins are sufficiently pure and are sufficiently free from other biological constituents of their

hosts cells so as to be useful in, for example, protein sequencing, or producing pharmaceutical preparations.

In another set of embodiments an isolated nucleic acid encoding the substantially pure polysaccharide lyase of the invention is provided. As used herein with respect to nucleic acids, the term "isolated" means: (I) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art.

As used herein, a coding sequence and regulatory sequences are said to be "operably joined" when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were

capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Promoters may be constitutive or inducible. Regulatory sequences may also include enhancer sequences or upstream activator sequences, as desired.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids and phagemids. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium, or just a single time per host as the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose

activities are detectable by standard assays known in the art (e.g., ß-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques. Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, the term "stringent conditions" refers to parameters known to those skilled in the art. One example of stringent conditions is hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin (BSA), 25mM NaH_2PO_4 (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecylsulphate; and EDTA is ethylene diamine tetra acetic acid. There are other conditions, reagents, and so forth which can be used, which result in the same degree of stringency. A skilled artisan will be familiar with such conditions, and thus they are not given here. The skilled artisan also is familiar with the methodology for screening cells for expression of such molecules, which then are routinely isolated, followed by isolation of the pertinent nucleic acid. Thus, homologs and alleles of the substantially pure polysaccharide lyases of the invention, as well as nucleic acids encoding the same, may be obtained routinely, and the invention is not intended to be limited to the specific sequences disclosed.

For prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host may be used. Examples of suitable plasmid vectors include pBR322, pUC18, pUC19 and the like; suitable phage or bacteriophage vectors include λgt10, λgt11 and the like; and suitable virus vectors include pMAM-neo, pKRC and the like. Preferably, the selected vector of the present invention has the capacity to autonomously replicate in the selected host cell. Useful prokaryotic hosts include bacteria such as *E. coli, Flavobacterium heparinum, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia,* and the like.

To express the substantially pure polysaccharide lyases of the invention in a prokaryotic cell, it is necessary to operably join the substantially pure polysaccharide lyases of the invention

sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage λ, the *bla* promoter of the β-lactamase gene sequence of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), the *trp, recA, lacZ, lacI,* and *gal* promoters of *E. coli,* the α-amylase (Ulmanen et al., *J. Bacteriol.* 162:176-182 (1985)) and the ζ-28-specific promoters of *B. subtilis* (Gilman et al., *Gene sequence* 32:11-20 (1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: *The Molecular Biology of the Bacilli,* Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward et al., *Mol. Gen. Genet.* 203:468-478 (1986)).

Prokaryotic promoters are reviewed by Glick *(J. Ind. Microbiol.* 1:277-282 (1987)); Cenatiempo *(Biochimie* 68:505-516 (1986)); and Gottesman *(Ann. Rev. Genet.* 18:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold et al. (*Ann. Rev. Microbiol.* 35:365-404 (1981)).

Because prokaryotic cells will not produce the substantially pure polysaccharide lyases of the invention with normal eukaryotic glycosylation, expression of the substantially pure polysaccharide lyases of the invention of the invention by eukaryotic hosts is possible when glysoylation is desired. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, and mammalian cells, either *in vivo* or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/0-AG14 or the myeloma P3x63Sg8, and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as neuroblastoma cell lines such as IMR 332 that may provide better capacities for correct post-translational

processing. Embryonic cells and mature cells of a transplantable organ also are useful according to some aspects of the invention.

In addition, plant cells are also available as hosts, and control sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences.

Another preferred host is an insect cell, for example in *Drosophila* larvae. Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used (Rubin, *Science* 240:1453-1459 (1988)). Alternatively, baculovirus vectors can be engineered to express large amounts of the substantially pure polysaccharide lyases of the inventionin insects cells (Jasny, *Science* 238:1653 (1987); Miller et al., In: *Genetic Engineering* (1986), Setlow, J.K., et al., eds., Plenum, Vol. 8, pp. 277-297).

Any of a series of yeast gene sequence expression systems may also be utilized which incorporate promoter and termination elements from the genes coding for glycolytic enzymes which are produced in large quantities when the yeast are grown in media rich in glucose. Known glycolytic gene sequences can also provide very efficient transcriptional control signals. Yeast provide substantial advantages in that they can also carry out post-translational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognize leader sequences on cloned mammalian gene sequence products and secrete peptides bearing leader sequences (i.e., pre-peptides).

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that

expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or which are subject to chemical (such as metabolite) regulation.

As discussed above, expression of the substantially pure polysaccharide lyases of the invention in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the promoter of the mouse metallothionein I gene sequence (Hamer et al., *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* 31:355-365 (1982)); the SV40 early promoter (Benoist et al., *Nature (London)* 290:304-310 (1981)); the yeast *gal4* gene sequence promoter (Johnston et al., *Proc. Natl. Acad. Sci. (USA)* 79:6971-6975 (1982); Silver et al., *Proc. Natl. Acad. Sci. (USA)* 81:5951-5955 (1984)).

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the substantially pure polysaccharide lyases of the invention does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as the substantially pure polysaccharide lyases of the invention coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the substantially pure polysaccharide lyases of the invention coding sequence).

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may, for example, provide for prototrophy to an auxotrophic host or may confer biocide resistance to, e.g., antibiotics, heavy metals, or the like. The selectable marker gene sequence can either be directly

linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of the substantially pure polysaccharide lyases of the invention mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, *Molec. Cell. Biol.* 3:280 (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColEl, pSC101, pACYC 184, and πVX. Such plasmids are, for example, disclosed by Sambrook, et al. (*Molecular Cloning: A Laboratory Manual,* second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989)). *Bacillus* plasmids include pC194, pC221, pT127, and the like. Such plasmids are disclosed by Gryczan (In: *The Molecular Biology of the Bacilli,* Academic Press, NY (1982), pp. 307-329). Suitable *Streptomyces* plasmids include pIJ101 (Kendall et al., *J. Bacteriol.* 169:4177-4183 (1987)), and streptomyces bacteriophages such as ϕC31 (Chater et al., In: *Sixth International Symposium on Actinomycetales Biology,* Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John et al. (*Rev. Infect. Dis.* 8:693-704 (1986)), and Izaki (*Jpn. J. Bacteriol.* 33:729-742 (1978)).

Preferred eukaryotic plasmids include, for example, BPV, EBV, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art (Botstein et al., *Miami Wntr. Symp.* 19:265-274 (1982); Broach, In: *The Molecular Biology of the Yeast Saccharomyces:*

Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, *Cell* 28:203-204 (1982); Bollon et al., *J. Clin. Hematol. Oncol.* 10:39-48 (1980); Maniatis, In: *Cell Biology: A Comprehensive Treatise,* Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608 (1980)). Other preferred eukaryotic vectors are viral vectors. For example, and not by way of limitation, the pox virus, herpes virus, adenovirus and various retroviruses may be employed. The viral vectors may include either DNA or RNA viruses to cause expression of the insert DNA or insert RNA. In addition, DNA or RNA encoding the substantially pure polysaccharide lyases of the invention polypeptides may be directly injected into cells or may be impelled through cell membranes after being adhered to microparticles (see below).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, i.e., transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, and the like. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the substantially pure polysaccharide lyases of the invention. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like).

The foregoing written specification is to be considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the particular examples disclosed herein as these embodiments are intended only as illustrations of the aspects of the invention and any recombinant polysaccharide lyases that are functionally equivalent are within the scope of the invention. Therefore, any sequences that are functionally equivalent of those described herein are within the spirit and scope of the claims appended hereto. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description.

Example 1

Purification of heparinase I: Lyophilized powdered extracts of F. heparinum were prepared according to the method of Yang et al. (1985). Partially purified heparinase I was prepared by step gradient elution of powdered cell extracts from a hydroxyapatite column followed by reverse adsorption on a QAE-Sephadex column. Further purification of the enzyme was accomplished with a chromatofocusing, 1 cm x 18 cm column with PBE resin (Pharmacia). A 1.5-2.5 ml aliquot of protein (0.5-1.5 mg) in 0.01 M phosphate (pH 6.8) was applied to the column equilibrated with 0.025 M ethanolamine (pH 9.4 with acetic acid). Elution was carried out with 150 ml of 10% Polybuffer 96 (pH 6.0 with acetic acid). The chromatofocused heparinase I eluted between 10 and 15 ml (pH 8.0-8.2). Fractions (1.5-2.0 ml) were collected in tubes containing 0.2 ml of 0.5 M phosphate buffer at pH 6.8. Immediately after collection, the fractions were assayed for heparinase I activity. Prior activity determination in the presence of Polybuffer showed no inhibition or interference by the ampholytes. The active fractions were pooled, and NaCl was added from a 2.0 M aqueous solution to give a final concentration of 0.1 M. Heparinase I was concentrated and equilibrated with a buffer consisting of 0.01 M NaH_2PO_4 and 0.1 M NaCl at pH 6.8 (PBS) with Centricon P-30 microconcentrators (Amicon, MA). The recovery of activity from the column was up to 90%. The resulting enzyme, equilibrated with PBS, is stable for up to 5 days at 4°C.

Heparinase I was purified to homogeneity by reverse-phase high pressure liquid chromatography with an HP 1090 (equipped with a diode array detector for multiple wavelengths, an on-line chart recorder monitoring 210 and 277 nm wavelengths) on a Vydac C_{18} reverse-phase column. The enzyme was eluted with a gradient of 0 to 80% acetonitrile in 0.1% TFA for 120 min. Heparinase I appeared as a doublet. Both the major and minor peaks had similar UV and tryptic digest profiles (Sasisekharan, 1991). The major peak was used throughout this work. It is believed that the isoforms of heparinase I are due to some unknown post-translational modification (Zimmermann, 1989). However, the labeling results were unaffected for samples in which the two peaks were not clearly resolved (Sasisekharan, 1991).

The enzyme was inactive following reverse phase HPLC. Protein concentrations were determined using Micro BCA reagent (Pierce Inc., IL) relative to a bovine serum albumin standard. To determine the purity and homogeneity of heparinase I, mass spectrometry was performed on a Laser MAT (Finnigan, CA) (Sasisekharan et al., 1994). Amino acid composition analysis was performed on an amino acid analyzer (Model 420, Applied Biosystems, CA) in Biopolymers Laboratory, Center for Cancer Research, MIT.

Heparinase was radio-iodinated with the Enzymobead reagent (BioRad), followed by removal of unbound [^{125}I] by passage over a PD-10 column (Pharmacia) equilibrated with the appropriate buffer. The radio-labeled enzyme was diluted with unlabeled enzyme. The specific activity of the protein ranged 10^4-10^5 cpm/mg.

Example 2

Pyridylethylation of cysteines: Pyridylethylation is a cysteine modification method that alkylates the cysteine using 4-vinyl pyridine (4-VP) (Andrews & Dixon, 1987). The alkylating group, 4-VP, is a hydrophobic residue that is stable in modified cysteines. The 4-VP modified cysteine(s) can be characterized easily by amino acid analysis. In this work the amino acid analyses on the 4-VP cysteine heparinase I indicated the presence of 2.14 ± 0.2 cysteines. There was no increase in pyridylethyl cysteine content following treatment with DTT demonstrating the absence of any disulfide bonds.

Example 3

Radio-labeling and tryptic mapping of heparinase I: Heparinase I which had been treated with 2 mM [^{3}H]iodoacetic acid in the presence of guanidine hydrochloride and DTT had ~ 0.55 ± 0.05 x 10^4 cpm [^{3}H]iodoacetic acid/μg of heparinase or 2.2 ± 0.05 x 10^5 cpm [^{3}H]iodoacetic acid/nmole of heparinase. Physical mapping of the cysteines of heparinase I was performed by peptide mapping with trypsin, followed by amino acid sequencing. The [^{3}H] labeled peptides

were peak 7 or Cys135, peak 65 or Cys297, and a third peak: peptide 61 which also turned out to be Cys297. The sequences of the three peptides corresponding to these peaks were:

Peak 7	K G I C* E Q G S S R
Peak 65	K M P F A Q F P K D I C* W I T F D V A I D W T K
Peak 61	K D I C* W I T F D V A I D W T K

The above results show that mature heparinase I from F. heparinum contains two free cysteines and not a disulfide bridge.

Example 4

Modification with Organomercurials: The reversible, sulfhydryl specific anion, PCMB, was utilized to determine the effects of sulfhydryl modification on heparinase I activity. Chromatofocused heparinase I treated with PCMB at 2.5-100 μM and 4°C resulted in a reversible loss of 95 ± 5% of enzyme activity. Upon addition of 10 mM DTT, up to 90% of the lost enzyme activity is recovered within 1 h at 4°C, verifying the sulfhydryl specificity of the reaction. Overnight incubations did not result in further inactivation of the enzyme, and 90% of the activity was recovered subsequently by treatment with 10 mM DTT. The time course of inactivation in the presence and absence of heparin also was determined. In the presence of 0.5 mg/ml heparin (~5 x K_m), the rate of inactivation was significantly decreased. Rate constants were determined by assuming pseudo first order kinetics and fitting of the data to the equation:

$$A_t = A_o \exp(-t/\tau) + A_\infty$$

where A_t is the fractional activity at time t, A_o is the initial fractional activity, A_∞ is the residual activity at infinite time, and τ is the inactivation time constant in minutes or the reciprocal rate constant. Time constants were obtained by a nonlinear least squares fit of the data to the equation. The best fit parameters obtained in the absence of heparin were $A_o = 0.72 \pm 0.05$, $A_\infty =$

0.25 ± 0.03, and $\tau = 0.8 \pm 0.1$ min. In the presence of 0.5 mg/ml heparin, the best fit parameters were $A_o = 0.67 \pm 0.08$, $A_\infty = 0.28 \pm 0.03$, and $\tau = 5 \pm 1$ min. The corresponding rate constants with 0.0 and 0.5 mg/ml heparin are 1.2 min^{-1} and 0.2 min^{-1}, respectively. Thus the presence of heparin reduces the inactivation rate 6-fold. The heparin concentration in the assay medium (25 mg/ml) was much larger than the K_m of 0.1 mg/ml (Yang et al., 1985); consequently, any additional heparin introduced did not alter the kinetics.

The use of PCMB-sulfonic acid resulted in similar inactivation behavior. PCMBS treatment was carried out following reduction with DTT in order to ascertain whether the modifications of any exposed sulfhydryls affected the activity. HeparinaseI pretreated with 1 mM DTT for 4 h at 4°C under nitrogen followed by modification with 2.5 mM PCMBS resulted in the same reaction kinetics as observed with untreated heparinase. Heparin was not included in these experiments. Further, similar heparinase inactivation results were obtained when recombinant heparinase I was used in the PCMB labeling studies.

Example 5

N-ethylmaleimide Derivatization: Different sulfhydryl reagents often exhibit disparate reactivities with proteins due to protein structural properties (Vallee & Riordan, 1969). The effect of NEM modification on heparinase I activity was examined in an attempt to elucidate structural influences on the cysteine reactivities. Treatment of heparinase I with 1 mM NEM at pH 7.0, showed little change in activity over an 8 h period. Heparinase I treated with 1 mM NEM overnight at 4°C, result in an activity loss of about 15%. In contrast, in the presence of 10 mM NEM pH 7.0 at 4°C, 15% of the enzymatic activity was lost within 45 min.

In order to ascertain whether significant levels of NEM bind heparinase I, labeling studies with [^{3}H]NEM were undertaken. Non-denatured heparinase incubated with 1 mM [^{3}H]NEM for 8 h incorporates 0.3×10^{-9} nmole [^{3}H]NEM/μg heparinase I, as determined from radio-labeled heparinase I electroeluted from an SDS gel. If the reaction was allowed to proceed for 20 h, up to 1×10^{-9} nmole [^{3}H]NEM/μg heparinase I was incorporated with a corresponding 15% loss of

activity. In contrast, if the enzyme was first denatured by incubation with either 0.1% SDS or 5 M guanidine hydrochloride, about 6 x 10^{-9} nmole [^{3}H]NEM/μg heparinase I was incorporated within an 8 h incubation at 4°C.

Example 6

Derivatization by Iodoacetamide and Iodoacetic Acid: The effect of reagent charge on cysteine reactivity was investigated by use of the negatively charged reagent iodoacetic acid and its neutral analogue, iodoacetamide. In addition to their charge difference, the reactivity of iodoacetamide is 5-7 times faster than iodoacetic acid with free cysteine in aqueous media (MacQuarrie & Bernhard, 1971).

Heparinase I incubated with 2, 5, 10, and 120 mM iodoacetamide in PBS at pH 7.0 and 4°C for up to 24 h exhibited little change in activity. A 15% inactivation occurred only after a 24 h incubation in the presence of 120 mM iodoacetamide. Iodoacetamide, therefore, does not significantly modify heparinase I.

In contrast, in the presence of 2 mM iodoacetic acid in PBS at 4°C, 95 ± 5% of heparinase I was inactivated within 10 min. The inactivation rate was concentration dependent: at 1 mM and 0.1 mM iodoacetic acid, inactivation was complete within 15 min and 15 h, respectively. The sensitivity of the iodoacetic acid binding site to the presence of heparin was demonstrated by the decrease in the inactivation rate in 500 mM iodoacetic acid from 3 ± 1 x 10^{-3} h^{-1} to 5 ± 2 x 10^{-4} h^{-1} in the presence of 2 mg/ml heparin. The retention of activity relative to untreated heparinase I was 15 ± 5%. Pretreatment of the enzyme for 4 h at 4°C with DTT under nitrogen had no effect on the modification.

It is known that the iodoacetic acid reactive form of cysteine is the mercaptide anion, and that the reaction rate increases with increasing pH (Torchinsky, 1981). In particular, the relative free cysteine alkylation rates at pH 5.6, pH 7.02, and pH 8.36 are 0.14, 1.0, and 2.1, respectively (Torchinsky, 1981). If the heparinase I cysteine were unaffected by the presence of nearby amino acids in the protein, the iodoacetic acid inactivation rates at 6.5 and 8.0 would be expected to

vary by an order of magnitude. The pseudo first order heparinase I inactivation rate constants obtained at pH 6.5, 7.0, 7.5, 8.0 and 8.5, however, varied very little over the pH range. The rate constants, k, were determined from the activity after a determined time, A_t, the initial activity A_o, the residual activity at infinite time, A_∞, and the equation:

$$k = (1/\tau) \ln [(A_t - A_\infty)/A_0]$$

The small variation in the rate of inactivation suggests that the cysteine was activated by the presence of nearby basic amino acids (Hammond & Gutfreund, 1959, Rabin & Watts, 1960).

The above results, i.e the marginal modification of heparinase I by iodoacetamide and the significant inactivation of heparinase I by iodoacetic acid, corroborates with the hypothesis that the environment around the cysteine residue is basic.

Example 7

Salt dependence of PCMB labeling: In order to show that the positive environment around the PCMB-reactive cysteine influences the labeling of the negatively charged PCMB, cysteine labeling by PCMB was performed under different salt concentrations and the time course of inactivation of heparinase I by PCMB with increasing salt concentrations was calculated. Heparinase I inactivation rate by PCMB was significantly reduced with increasing salt concentration 50, 100 and 200 mM NaCl. Indeed, no inactivation was detectable at a salt concentration of 200 mM NaCl. This result is consistent with the observation that the environment around the cysteine is positively charged, and the alteration of the electrostatic properties of this region by changing the salt concentration has a significant effect on the rate of the PCMB based inactivation of the thiol group. Thus, this result supports the conclusion that the environment around the PCMB-reactive cysteine is basic.

Example 8

Mapping the Modified Cysteine: In order to further verify that the PCMB-reactive cysteine was at the same site as the iodoacetic acid binding cysteine, [^{3}H]iodoacetic acid was reacted with nondenatured PCMB-modified heparinase I, and the amount of label incorporated was compared to that of nondenatured, untreated heparinase I labeled with [^{3}H]iodoacetic acid under identical conditions. In these experiments, it was found that the [^{3}H]iodoacetic acid labeling was reduced by 80% following treatment with PCMB. This suggested that the iodoacetic acid binding site and the PCMB binding site are identical. This was conclusively determined by peptide mapping by trypsin digestion and amino acid sequencing of the modified heparinase I cysteine.

The PCMB-labeled heparinase I was isolated, denatured and then reacted with iodoacetamide to block the other cysteine. Following this, the enzyme was treated with DTT to remove the bound PCMB, and then labeled with [^{3}H]iodoacetic acid. Modified heparinase I was digested with trypsin and the tryptic peptides were separated. Only one cysteine, Cys135, was selectively labeled by [^{3}H]iodoacetic acid. Cys297 was not labeled by [^{3}H]iodoacetic acid in this experiment. In another experiment, heparinase I was first labeled at the reactive cysteine with PCMB. The enzyme was then denatured, labeled with [^{3}H]iodoacetic acid, and re-chromatographed to remove the excess radiolabel. Following this, the enzyme was digested with trypsin and the tryptic peptides were separated by RP-HPLC. In this experiment Cys297 was selectively [^{3}H] labeled, while Cys135 was not. The results of the above experiments taken together, confirm that Cys135 is the PCMB-labeled or the active site cysteine. In addition, [^{3}H]iodoacetic acid labeling had little or no cross-reactivity and was selective in labeling the cysteines.

Example 9

Cysteine-Modified Recombinant Heparinase: Site-directed mutations were performed to confirm the role of Cys135 in heparinase I activity. Seven mutant recombinant heparinases were designed: C135S (Cys135 to serine conversion), C135H (Cys135 to histidine conversion), C135E (Cys135 to glutamate conversion), C135D (Cys135 to aspartate conversion), C135A (Cys135 to

alanine conversion), C297S (Cys297 to serine conversion), and C297A (Cys297 to alanine conversion).

Recombinant polysaccharide lyases were produced as soluble protein in BL21DE3 E.coli host, using the pET 15b system, where expression is driven by bacteriophage T7 Polymerase. This construct has a histidine tag (6 consecutive histidines), which constitutes a high affinity site for Ni^{2+}, and a thrombin cleavage site in a 21 amino acid N-terminal leader sequence. The expression is induced by IPTG (isopropyl-β-D-thiogalactoside). The *-L* heparinase construct starts with a sequence which reads Met, Gln22, Gln23, Lys24, Lys25, Ser26 .... (Sasisekharan et al., 1993). The Met residue was added before the Gln22 to introduce a start codon. The Cys135 and Cys297 mutations were introduced as part of PCR primers. These primers, together with the T7 promotor (5') and T7 terminator (3') primers (Novogen, WI) (which flank the heparinase gene), were used to create two PCR products that overlap in sequence, with the *-L* heparinase gene construct as a template. The overlapping products were isolated from low melt agarose (SeaPlaque, FMC, or GIBCO BRL, Gaithersburg, MD), denatured (100°C), and allowed to reanneal (room temperature) to produce two possible heteroduplex products (Higuchi, 1990). The heteroduplex with the recessed 3' ends was filled-in using Taq polymerase. This fragment was used as a template in a 12 cycle PCR (Higuchi, 1990) with the 5' and the 3' primers respectively. The PCR product was isolated from a low melt gel and ligated overnight directly into T-vectors (Marchuk, et al., 1991). T-vector was prepared as described in Marchuk et al., 1991. Briefly, pBluescript (Stratagene, LaJolla, CA) was digested with EcoRV (New England Biolabs, Beverly, MA) and isolated and gel purified from a low melt gel. The purified linear plasmid was then incubated with Taq polymerase (1unit/μg plasmid/20 μl volume) (Perkin Elmer, Norwalk, CT) and 2mM dTTP for 2 hrs at 70°C, using standard buffer conditions. The sub-cloned heparinase I PCR fragments were excised from T-vector by digestion with NdeI and BamHI, gel purified, and then ligated into pET-15b plasmid (predigested at the NdeI and BamHI sites and gel purified) using T4 DNA ligase (New England Biolabs, MA). The ligation mixture then was used to transform DH5α competent cells (GIBCO BRL). The plasmid containing the

recombinant lyase gene in pET-15b was isolated, purified using Miniprep (Qiagen, Charsworth, CA), and used to transform the host cell BL21(DE3) (Novogen, WI). Recombinant heparinase I construct devoid of the putative signal sequence (-L r-heparinase I) was also expressed as a control (Sasisekharan et al., 1993).

The constructs were transformed in BL21(DE3) (Novagen), grown overnight, diluted in 100 ml LB, 250 μg/ml ampicillin and grown to an OD_{600} of 0.5. The culture was induced with 1 mM IPTG for 2 hours, harvested by centrifugation (4°C, 3500g x 10 min), washed in cold phosphate buffered saline (PBS) and resuspended in 1/20th volume binding buffer (20 mM Tris, 500 mM NaCl, 5 mM Imidazole). The resuspended culture was placed in an ice bath, sonicated for 2 min using a Branson 450 sonicator (Branson, Danbury, CT) (power 3, 50% pulse) and centrifuged at 4°C and 15,000 g for 30 min. The supernatant was assayed for activity and purified by Ni^{2+} affinity chromatography using sepharose 6B Fast Flow resin covalently linked to nitrilotriacetic acid (Novogen, WI). Briefly, the resin was charged with 5 column volumes 200 mM $NiSO_4$ and equilibrated with 5 column volumes binding buffer. Then, 6-10 ml sample was applied followed by 12 ml binding buffer, 9 ml 15% elution buffer (20 mM Tris, 500 mM NaCl, 200 mM Imidazole) and 10 ml 100% elution buffer. The enzymes were recovered in 4 ml of the 100% elution step, desalted on two PD10 columns (BioRad, Richmond, CA) and incubated overnight at 4°C with 0.5 units thrombin (Novagen, WI). Cleaved enzymes were applied to the stripped (20 mM Tris, 500 mM NaCl, 100 mM EDTA) and regenerated column and collected in the flow through fraction. SDS-PAGE (Laemmli, 1970) was carried out using precast 12% gels and a Mini Protean II apparatus, and stained with the Silver Stain Plus kit.

The level of protein expression for all the recombinant heparinases was identical in the BL21(DE3) host. While -L r-heparinase I control was expressed as a soluble protein in E. coli with an activity of ~ 5.2 U/mg of E. coli crude extract (Sasisekharan et al., 1993), the C135A r-heparinase I was expressed in BL21(DE3) with no enzymatic activity. Interestingly, the C135S r-heparinase I was expressed in BL21(DE3) with an activity of ~ 0.06 U/mg of E. coli crude extract. The C135H, C135E, and C135D recombinants show ~50% of the activity of the -L r-

heparinase. Importantly, the mutations at Cys297 (C297S and C297A) were both expressed in the same host with no change in their enzymatic activity compared to the -L r-heparinase I control.

The r-heparinase degradation of heparin was identical to that of the purified F. heparinum heparinase I, producing the two di-, the three tetra-, and the hexasaccharides. The above results taken together show that Cys135 is important for heparinase I activity and that altering Cys297 did not alter heparinase I activity.

Example 10

Calcium Dependence of Heparinase I Activity: Heparinase I samples were extensively desalted using Centricon P-30 microconcentrator to remove residual calcium from the hydroxylapatite step during the enzyme purification (Yang, et al., 1985; Sasisekharan et al., 1994). The heparin concentration was fixed at 25 mg/ml in all experiments, and only the calcium concentration was varied. Activity was seen to increase with calcium concentrations increasing up to about 5-10 mM. A region of heparinase I (residues 206-213) was found to be homologous to the calcium binding loop of the EF-hand structural domain (Kretsinger et al., 1991). Of the five amino acids that are involved in coordinating calcium, four are conserved in heparinase I (Table II). Also the glycine and hydrophobic residue at the top of the loop are conserved. This suggested a calcium coordinating site in heparinase I.

Example 11

Heparin Affinity Chromatography: The affinity separation of heparinase I was carried out in the presence and absence of calcium. Heparinase I was seen to bind to heparin-POROS, and the bound enzyme could be eluted at a salt concentration of about 200 mM. The protein eluted as a doublet, consistent with results from heparinase I purification (Sasisekharan, et al., 1993). When the affinity separation was carried out in the presence of calcium (5mM), heparinase I

eluted in the void volume since the enzyme cleaves the heparin to which it binds (this was confirmed by the appearance of oligosaccharide products in the void volume).

Example 12

Affinity Co-electrophoresis: Affinity co-electrophoresis (ACE) was used to quantify heparin binding to heparinase I. The technique measures the extent of binding based on the retardation of heparin when electrophoresed in the presence of heparinase I embedded in an agarose gel. ACE was carried out in the presence or absence of iodoacetic acid to determine the importance of the active site Cys135 in the binding of heparin to heparinase I and in the absence of calcium to prevent heparin degradation. At a sufficiently high enzyme concentration, the migration of heparin is retarded in a dose-dependent manner. There is no difference in the retardation of heparin for the iodoacetic acid modified heparinase I when compared to the unmodified heparinase I. This result indicates that blocking the active site cysteine does not alter heparin binding.

To determine a binding constant, a Scatchard plot was obtained by plotting R/C vs. R, where R is the retardation coefficient $R= (M_0 -M)/M_0$, M_0 is the mobility of free heparin, and M is the observed heparin mobility in a zone with protein concentration of C. Assuming a single site, bimolecular association, the data were fitted to a straight line with a slope of $-1/K_d$ (Lee and Lander, 1991). The dissociation constant for heparinase-heparin binding was found to be 60 nM by this technique. Furthermore, an ACE gel of heparin-heparinase carried out in the presence of calcium showed extensive smearing of the heparin band, since heparinase I cleaved heparin in the presence of calcium. No heparin retardation could be observed on this gel.

Example 13

Heparin Blotting of CnBr Digests of Heparinase I: CnBr digested heparinase I separated by SDS-PAGE resulted in 10 peptide fragments. Heparinase I contains 5 internal methionine residues (CnBr sites) two of which are adjacent, so for complete digestion, only 5 fragments

should be expected. Only 4 of the smaller peptides could be sequenced as the larger fragments, based on molecular weights and sequencing, probably represented partial digests from the N-terminus which previously was shown to be blocked (Sasisekharan, et al., 1993). The CnBr digested heparinase I fragments were transferred onto nitrocellulose and hybridized with labeled heparin, and counted for ^{125}I incorporation. The binding of ^{125}I-heparin to one peptide band (CnBr-8), was 2-4 times as high as binding to the other bands and to controls. Similar results were obtained by an alternative method where the peptide bands were cut out and then hybridized individually. CnBr-8 is a partial digest of approximately 10 kDa, spanning amino acids 196 to approximately 290 of the heparinase I primary sequence. It has a lysine rich N-terminal region, containing two Cardin-Weintraub heparin binding consensus sequence and a calcium binding loop of the EF-hand structural domain. CnBr-7 is ~13-kDa, spanning amino acids 272 to approximately 360. The region from 272 to 290 is common to CnBr-7 and CnBr-8 and, since CnBr-7 did not bind heparin, it is thus excluded from being a part of the heparin binding domain. These results indicates the region 195-270 contains the primary heparin binding site and that this site is still functional in the isolated CnBr-8 peptide. To further narrow down the heparin-binding region, we performed tryptic digests which cleave heparinase I to much smaller fragments than CnBr.

Example 14

Competitive Binding and Dot-blots with Tryptic Digests of Heparinase I: Tryptic mapping of heparinase I has been standardized using RP-HPLC (Sasisekharan, et al., 1993). Even though heparinase I is a very basic protein (having a pI of 9.1), it binds very well to a hydrophobic surface as its elutes at a relatively high acetonitrile concentration of 72% in RP-HPLC. Interestingly, we found that heparin, but not chondroitin sulfate, was able to prevent heparinase I binding to a reverse-phase column in a concentration dependent manner.

We tested the ability of heparin in protecting the heparin binding domain of heparinase I from trypsin cleavage. Under the conditions tested, we observed that heparin was ineffective in

protecting the heparin binding domain but, nonetheless, it was able to specifically compete with the binding of some heparinase I tryptic peptides to the reverse phase column. Peaks that shifted significantly in their elution time, or disappeared (presumably eluting in the void volume), represent tryptic peptides that binds to heparin. Chondroitin sulfate was used as a control to account for non-specific ionic effects of heparin on elution of the peptides. Compared to a control tryptic map, no significant changes were observed in the tryptic digests performed in the presence of increasing concentration of chondroitin sulfate except for the appearance of a peak about 42 min and the overall diminishing of peak sizes for td9, those eluting between 52-58 mins, and td50. However, in the presence of increasing concentrations of heparin, the following peaks were altered reproducibly: td4, td9, peaks eluting between 52-58 mins td39, td 45, td 50. As the peptides td9, those eluting in the region between 52-58 mins, and td50 were altered by both heparin and chondroitin sulfate, it is probable that these peptides non-specifically interact with these acidic polysaccharides. However, in the presence of heparin alone (or heparin with chondroitin sulfate) td4, td39 and td45 were absent (from the region where they should elute) in the tryptic map, indicating specific binding to heparin. In a dot blot assay, for specific binding of ^{125}I heparin to heparinase I tryptic peptides, in the presence of a 100 fold excess of cold chondroitin sulfate, only td45 showed ^{125}I signal. In addition, there were ^{125}I signals near the isocratic region of the chromatogram where di- and tri-peptides, containing Lys and Arg residues, elute.

The sequences of tryptic peptides from heparinase I are given in Table III. It can be concluded that td45 (residues 215-221) and td4 (residues 132-141) are the only peptides from the tryptic digest experiments that bind specifically to heparin; consistent with td39 being a part of CnBr-8 peptide, and td4 being a part of the active site of heparinase I. The combined heparin binding results from experiments with CnBr and tryptic digests of heparinase I points to the region of residues 195-221 as being directly involved in heparin binding. Importantly, the region from 195-220 contain multiple lysines and is likely to be degraded to very short peptides (di and

tripeptides) by trypsin. Thus, it would not have been expected to show up on the tryptic digest chromatogram.

Example 15

Synthetic Heparin Binding Domain of Heparinase I: The region 196-213 was synthesized as a peptide (HBP-I). The peptide (HBP-I) has a ~4 micromolar binding affinity for heparin dodecasaccharides. Interestingly, HBP-I affected the product profile of heparinase I degradation of heparin. As mentioned earlier, heparinase depolymerization of heparin results in two disaccharides, three tetrasaccharides (1-3), and a hexasaccharide. In a concentration dependent manner, the addition of HBP-I to the reaction mixture caused the peak corresponding to tetrasaccharide 3 ($\Delta U_{2S} H_{NS,6S} I_{2S} H_{NS,6S}$) to disappear. When tetrasaccharide 3 was isolated and degraded with heparinase I in the presence of HBP-I, a marked increase in the amount of disaccharide was observed. A control peptide with similar charge properties (and at the concentration ranges tested above) had no effect on the enzyme activity or on the oligosaccharide product profile. This demonstrates that HBP-I affects the selectivity of heparin degradation by heparinase I: Tetrasaccharide 3, but not tetrasaccharides 1 or 2, is degraded to a large extent in the presence of HBP-I.

Example 16

PCMB Protection and Tryptic Digest: Heparinase I derivitization by sulfhydryl specific reagent PCMB inactivated the enzyme due to selective modification of the active site cysteine or Cys135 (see above). Further, the inability to selectively label Cys135 using PCMB in the presence of heparin indicated the existence of a heparin binding site in close proximity to Cys135. To test this hypothesis, tryptic digestion of PCMB modified heparinase I (PCMB-heparinase I) was carried out to determine if PCMB was able to protect the heparin binding sequence from trypsin cleavage. The PCMB-heparinase I tryptic map was marked by the appearance of a new peak. The peptide corresponding to this new peak consisted of residues

200-209 of heparinase I. This result suggests that PCMB labeled Cys135 protects this lysine-rich peptide (the heparin binding sequence) from trypsin cleavage, when compared to a control digest where this peptide is not observed. This result is consistent with the notion of a heparin binding site in close proximity to the active site Cys135.

Example 17

Binding Domain-Modified Recombinant Heparinase: To further investigate the function of the heparin/calcium binding domain, a series of recombinant lyases were produced in which the binding domain was modified by site-directed mutagenesis. The recombinants were produced by the same method described above for cysteine mutants.

For example, a mutant with His203 to alanine conversion (H203A) was constructed and both the mutant and the wild type r-heparinase I were expressed in E.coli. -L r-heparinase I degradation of heparin was identical to that of the purified F. heparinum heparinase I, producing the two di-, the three tetra-, and the hexasaccharides described above. The H203A mutant, on the other hand was completely inactive. Estimated from the intensity of the purified bands, the combined yield of protein expression and purification is identical for both wild type and mutant heparinase. This result demonstrates that His203 is critically required for enzyme activity. In addition, the results strongly suggests that the heparin binding region around residue 203 is in close proximity to the scissile bond during catalysis.

As additional examples, recombinant polysaccharide lyases were produced by site-directed mutagenesis in which Lys198 and Lys199 were substituted by alanine (K198A, K199D), aspartate (K198D, K199D) and arginine (K198R, K199R). The replacement of the positively charged lysine with the positively charged arginine had no apparent effect on activity or product profile. Replacement of either lysine with the neutral alanine or negative aspartate resulted in product profiles in which disaccharide 1 and tetrasaccharides 2 and 3 were negligible and disaccharide 1 and tetrasaccharide 2 were reduced to approximately 50% of the abundance obtained with native heparinase I.

Other recombinant polysaccharides produced in accordance with the present invention are shown in Table IV along with indications of their k_{cat} values and activity after overnight incubation with heparin. These polysaccharide lyases are illustrative and not exhaustive of those enabled by the present disclosure.

TABLE I

Turn#	PB1	PB2	PB3
1	26-30 SGNIP 10	35-38 VQAD 11	
2	48-51 NKWV 12	53-56 VGIN 13	
3	67-70 LRFN 14	75-77 YRF	
4	107-110 TNDF 15	116-117 SV	120-122 NAQ
5	128-131 YHYG 16	140-143 SRSY 17	146-148 SVY
6	154-156 PDN	159-160 TI	164-167 WHGA 18
7	171-175 TLVAT 19	178-179 GE	181-184 KTLS 20
8	215-218 ITYV 21	225-227 WKV	
9	234-238 TLAFG 22		243-247 YFYIK 23
10	261-264 RNNA 24	265-267 NPE	
11	297-299 CWI	301-304 FDVA 25	
12	323-329 DVMMTY 26		338-341 AHIV 27

TABLE II

EF-hand homology	n	c	x	x	c	x	c	gly	x	h	c	x	x	c	n
Heparinase I	V_{206}	**E**	K	K	**D**	K	**D**	**G**	K	**I**	**T**	Y	V	**A**	G_{220}
Score	+	+			+		+	+		+	+			-	-

The table shows the central Ca^{2+} coordinating homology domain of EF-hands, with the functional amino acids bolded (Kretsinger, 1975).

"c" indicates the Ca^{2+} coordinating amino acids, D,N,S,T,E or Q.

"h" indicates amino acids with hydrophobic side groups, I, L or V.

"n" indicates nonpolar amino acids, I, L, V, M, F, Y, W.

"x" indicates any amino acid.

TABLE III

Peptides	Amino Acid Sequence
td 4	(K,R) G I C E Q G S S R
td 9	(K,R) T V Y H Y G K
td 9'	(K,R) T S T I A Y K
td 21	(K,R) F G I Y R
td 33	(K,R) A D I V N Q Q E I L I G R D D * G Y Y F K
td 39	(K,R) I T Y V A G K P N G N K V E Q G G Y P T L A F *
td 43	(K,R) M P F A Q F P K D C W I T F D V A I D * T K
td 40	(K,R) N L S G Y S E T A R
td 45	K N I A H D K V E K K
td 72	K T L S I E E F L A L Y D R
td 50	R S Y T F S V Y I P S S F P D N A T T I F A Q W H G A P S R T L V A T P E G E I K

The table shows the peptides from tryptic digest of heparinase I. The sequence begins (K,R) because trypsin cuts at either lysine or arginine residues. * represents amino acids that could not be determined.

TABLE IV		
MUTANT	Kcat (s^{-1})	Products (%wild type)
C135A	0	none
C135D	3.5	~50
C135E	3.8	~50
C135S	2	<10
C135H	3	~50
H203A	0	none
H203D	3.5	~10
H203S	3.2	~10
H203C	3.9	~10
KK198AA	4.1	~50
KK198DD	4.1	~50
KK198RR	54	equivalent
KK208AA	24	~70
KK208DD	54	equivalent
KK208RR	60	equivalent
N200A	48	equivalent
N200K	~0	none
K205A	22	equivalent
K205Y	~0	none
E207A	18	~70
D210A	50	equivalent
D212A	50	equivalent
G213A	20	~25
K214A	60	equivalent

Recomb hep	92	equivalent
Native hep	100	

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GODAVARTI, RANGANATHAN
SASISEKHARAN, RAMNATH
ERNST, STEFFAN
GANESH VENKATARAMAN
COONEY, CHARLES L
LANGER, ROBERT

(ii) TITLE OF INVENTION: RATIONALLY DESIGNED POLYSACCHARIDE LYASES DERIVED FROM HEPARINASE I

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.

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(D) STATE: MA

(E) COUNTRY: USA

(F) ZIP: 02210

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 38,349

(C) REFERENCE/DOCKET NUMBER: MO656/7014

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1379 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: DERIVED FROM FLAVOBACTERIUM HEPARINUM

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 173..1327

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 236..1324

(D) OTHER INFORMATION: /product= "MATURE PEPTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTTTTGGGA GCAAAGGCAG AACCATCTCC GAACAAAGGC AGAACCAGCC TGTAAACAGA 60

CAGCAATTCA TCCGCTTTCA ACCAAAGTGA AAGCATTTAA TACAATACCA GAATGTCGCA 120

TTTCCCTTTC AGCGTACTTT TTGGGTAAAT AACCAATAAA AACTAAAGAC GG ATG 175
Met
1

AAA AAA CAA ATT CTA TAT CTG ATT GTA CTT CAG CAA CTG TTC CTC TGT 223
Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe Leu Cys
5 10 15

TCG GCT TAC GCC CAG CAA AAA AAA TCC GGT AAC ATC CCT TAC CGG GTA 271
Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro Tyr Arg Val
20 25 30

AAT GTG CAG GCC GAC AGT GCT AAG CAG AAG GCG ATT ATT GAC AAC AAA 319
Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile Ile Asp Asn Lys
35 40 45

TGG GTG GCA GTA GGC ATC AAT AAA CCT TAT GCA TTA CAA TAT GAC GAT 367
Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp Asp
50 55 60 65

AAA CTG CGC TTT AAT GGA AAA CCA TCC TAT CGC TTT GAG CTT AAA GCC 415
Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu Leu Lys Ala
70 75 80

GAA GAC AAT TCG CTT GAA GGT TAT GCT GCA GGA GAA ACA AAG GGC CGT 463
Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala Gly Glu Thr Lys Gly Arg
85 90 95

ACA GAA TTG TCG TAC AGC TAT GCA ACC ACC AAT GAT TTT AAG AAA TTT 511
Thr Glu Leu Ser Tyr Ser Tyr Ala Thr Thr Asn Asp Phe Lys Lys Phe
100 105 110

CCC CCA AGC GTA TAC CAA AAT GCG CAA AAG CTA AAA ACC GTT TAT CAT 559
Pro Pro Ser Val Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr His
115 120 125

TAC GGC AAA GGG ATT TGT GAA CAG GGG AGC TCC CGC AGC TAT ACC TTT 607
Tyr Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr Thr Phe
130 135 140 145

TCA GTG TAC ATA CCC TCC TCC TTC CCC GAC AAT GCG ACT ACT ATT TTT 655
Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile Phe
150 155 160

GCC CAA TGG CAT GGT GCA CCC AGC AGA ACG CTT GTA GCT ACA CCA GAG 703
Ala Gln Trp His Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro Glu
165 170 175

GGA GAA ATT AAA ACA CTG AGC ATA GAA GAG TTT TTG GCC TTA TAC GAC 751
Gly Glu Ile Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp
180 185 190

CGC ATG ATC TTC AAA AAA AAT ATC GCC CAT GAT AAA GTT GAA AAA AAA 799
Arg Met Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys Lys
195 200 205

GAT AAG GAC GGA AAA ATT ACT TAT GTA GCC GGA AAG CCA AAT GGC TGG 847
Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly Trp
210 215 220 225

AAG GTA GAA CAA GGT GGT TAT CCC ACG CTG GCC TTT GGT TTT TCT AAA 895
Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser Lys
230 235 240

GGG TAT TTT TAC ATC AAG GCA AAC TCC GAC CGG CAG TGG CTT ACC GAC 943
Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu Thr Asp
245 250 255

AAA GCC GAC CGT AAC AAT GCC AAT CCC GAG AAT AGT GAA GTA ATG AAG 991
Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met Lys
260 265 270

CCC TAT TCC TCG GAA TAC AAA ACT TCA ACC ATT GCC TAT AAA ATG CCC 1039
Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr Lys Met Pro
275 280 285

TTT GCC CAG TTC CCT AAA GAT TGC TGG ATT ACT TTT GAT GTC GCC ATA 1087
Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr Phe Asp Val Ala Ile
290 295 300 305

GAC TGG ACG AAA TAT GGA AAA GAG GCC AAT ACA ATT TTG AAA CCC GGT 1135
Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn Thr Ile Leu Lys Pro Gly
310 315 320

AAG CTG GAT GTG ATG ATG ACT TAT ACC AAG AAT AAG AAA CCA CAA AAA 1183
Lys Leu Asp Val Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln Lys
325 330 335

GCG CAT ATC GTA AAC CAG CAG GAA ATC CTG ATC GGA CGT AAC GAT GAC 1231
Ala His Ile Val Asn Gln Gln Glu Ile Leu Ile Gly Arg Asn Asp Asp
340 345 350

GAT GGC TAT TAC TTC AAA TTT GGA ATT TAC AGG GTC GGT AAC AGC ACG 1279
Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser Thr
355 360 365

GTC CCG GTT ACT TAT AAC CTG AGC GGG TAC AGC GAA ACT GCC AGA TAGCAAAAGC
1334
Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg
370 375 380 385

CCTAAGCGCA TCCGATAGGG CTTTTCTTAT ATTTACAATA AAATT 1379

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe Leu
1 5 10 15

Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro Tyr Arg
20 25 30

Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile Ile Asp Asn
35 40 45

Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp
50 55 60

Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu Leu Lys
65 70 75 80

Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala Gly Glu Thr Lys Gly
85 90 95

Arg Thr Glu Leu Ser Tyr Ser Tyr Ala Thr Thr Asn Asp Phe Lys Lys
100 105 110

Phe Pro Pro Ser Val Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr
115 120 125

His Tyr Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr Thr
130 135 140

Phe Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile
145 150 155 160

Phe Ala Gln Trp His Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro
165 170 175

Glu Gly Glu Ile Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr
180 185 190

Asp Arg Met Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys
195 200 205

Lys Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly
210 215 220

Trp Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser
225 230 235 240

Lys Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu Thr
245 250 255

Asp Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met
260 265 270

Lys Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr Lys Met
275 280 285

Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr Phe Asp Val Ala
290 295 300

Ile Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn Thr Ile Leu Lys Pro
305 310 315 320

Gly Lys Leu Asp Val Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln
325 330 335

Lys Ala His Ile Val Asn Gln Gln Glu Ile Leu Ile Gly Arg Asn Asp
340 345 350

Asp Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser
355 360 365

Thr Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg
370 375 380

Claims

We claim:

1. A substantially pure polysaccharide lyase comprising the amino acid sequence of the mature peptide of SEQ ID NO: 2 wherein at least one amino acid residue has been substituted and wherein

the substitution is selected from the group consisting of (a) a substitution of a cysteine residue corresponding to position 135 of SEQ ID NO: 2 with a residue selected from the group consisting of aspartate, glutamate, serine, threonine, and histidine; (b) a conservative substitution of a residue of a Cardin-Weintraub-like heparin-binding sequence XBBXXXBXB corresponding to positions 197-205 or 208-212 of SEQ ID NO: 2 with a residue which conforms to the heparin-binding sequence; (c) a conservative substitution of a residue of an EF-hand-like calcium binding sequence corresponding to positions 206-220 of SEQ ID NO: 2 with a residue which conforms to the calcium binding sequence; (d) a conservative substitution of a residue of a PB1, PB2 or PB3 β-sheet domain of SEQ ID NO: 2; (e) a non-conservative substitution of a cysteine residue corresponding to position 297 of SEQ ID NO: 2; (f) a non-conservative substitution of a residue of a PB1, PB2 or PB3 β-sheet domain of SEQ ID NO: 2 which preserves a parallel ß-helix tertiary structure characteristic of SEQ ID NO: 2;(g) a deletion of one or more residues of n N-terminal region or a C-terminal region of SEQ ID NO: 2 which preserves a parallel ß-helix tertiary structure characteristic of SEQ ID NO: 2; (h) a substitution of a histidine residue corresponding to position 203 of SEQ ID NO: 2 with a residue selected from the group consisting of aspartate, glutamate, serine, threonine and cysteine; (i) a substitution of a lysine residue corresponding to position 198, 199, 205, 208, 209, 211 or 214 of SEQ ID NO: 2 with a residue selected from the group consisting of the small non-polar amino acids, the small polar amino acids, and the acidic amino acids; (j) a substitution of a small polar or small non-polar amino acid for a residue corresponding to a position of SEQ ID NO: 2 selected from the group consisting of Phe197, Asn200, Asp204, Glu207, Asp210, Asp212 and Gly213; and (k) a non-conservative substitution of a serine residue corresponding to position 39 of SEQ ID NO: 2.

2. A substantially pure polysaccharide lyase as in claim 1 wherein the substitution comprises a substitution of a cysteine residue corresponding to position 135 of SEQ ID NO: 2 with a residue selected from the group consisting of aspartate, glutamate, serine, threonine, and histidine.

3 A substantially pure polysaccharide lyase as in claim 2 wherein at least a second amino acid residue has been substituted and wherein

the second substitution comprises a conservative substitution of a residue of a Cardin-Weintraub-like heparin binding sequence XBBXXXBXB corresponding to positions 197-205 of SEQ ID NO: 2 with a residue which conforms to the heparin binding sequence.

4 A substantially pure polysaccharide lyase as in claim 2 wherein at least a second amino acid residue has been substituted and wherein

the second substitution comprises a conservative substitution of a residue of an EF-hand-like calcium binding sequence corresponding to positions 206-220 with a residue which conforms to the calcium binding sequence.

5 A substantially pure polysaccharide lyase as in claim 2 wherein at least a second amino acid residue has been substituted and wherein

the second substitution comprises a conservative substitution of a residue of a PB1, PB2 or PB3 β-sheet domain of SEQ ID NO: 2.

6. A substantially pure polysaccharide lyase as in claim 1 wherein the substitution comprises a conservative substitution of a residue of a Cardin-Weintraub-like heparin binding sequence XBBXXXBXB corresponding to positions 197-205 of SEQ ID NO: 2 with a residue which conforms to the heparin binding sequence.

7. A substantially pure polysaccharide lyase as in claim 6 wherein the substitution comprises a substitution of a lysine residue corresponding to position 198, 199 or 205 of SEQ ID NO: 2 with an arginine or histidine.

8. A substantially pure polysaccharide lyase as in claim 6 wherein the substitution comprises a conservative substitution of a histidine residue corresponding to position 203 of SEQ ID NO: 2.

9. A substantially pure polysaccharide lyase as in claim 1 wherein the substitution comprises a conservative substitution of a residue of an EF-hand-like calcium binding sequence corresponding to positions 206-220 of SEQ ID NO: 2 with a residue which conforms to the calcium binding sequence.

10. A substantially pure polysaccharide lyase as in claim 9 wherein the substitution comprises a substitution of a lysine residue corresponding to position 208, 209, 211 or 214 of SEQ ID NO: 2 with an arginine or histidine.

11. A substantially pure polysaccharide lyase as in claim 9 wherein the substitution comprises a substitution of an aspartate residue corresponding to positions 210 or 212 of SEQ ID NO: 2 with a glutamate.

12. A substantially pure polysaccharide lyase as in claim 1 wherein the substitution comprises a substitution of a serine residue corresponding to position 39 of SEQ ID NO: 2 with a alanine residue.

13. A heparin fragment comprising:

a low moleuclar weight heparin fragment greater in size than a hexasaccharide obtainable by the process of incubating with heparin the substantially pure polysaccharide lyase of claim 1 to produce the high order low moleuclar weight heparin fragment.

14. A pharmaceutical preparation comprising a sterile formulation of the substantially pure polysaccharide lyase of claim 1 and a pharmaceutically acceptable carrier.

15. An isolated nucleic acid comprising

(a) an isolated nucleic acid encoding the substantially pure polysaccharide lyase of claim 1;

(b) nucleic acids which hybridize under stringent hybridization conditions to the nucleic acid of SEQ ID NO 1 or to the complement of the nucleic acid of SEQ ID NO 1 and which are modified to encode a modified heparinase as described in claim 1; and

(c) nucleic acids that differ from the nucleic acids of (b) in codon sequence due to the degeneracy of the genetic code.

16. A recombinant host cell including an isolated nucleic acid as in claim 15.

17. An expression vector including an isolated nucleic acid as in claim 15.

18. A substantially pure polysaccharide lyase comprising:

a modified heparinase having a modified heparinase k_{cat} value, wherein the modified heparinase k_{cat} value is $\leq 75\%$ of a native heparinase k_{cat} value of a complementary native heparinase.

19. An immobilized substantially pure modified heparinase comprising:

a modified heparinase as in claim 18, and

a solid support membrane, wherein the modified heparinase is immobilized on the solid support membrane.

20. A substantially pure polysaccharide lyase comprising:

a modified heparinase I having a modified product profile, wherein the modified product profile of the modified heparinase I is $\leq 50\%$ similar to a native product profile of a native heparinase I.

21. A substantially pure polysaccharide lyase comprising:

a modified heparinase I producing when contacted with heparin less than 20% of the disacharrides and trisaccharide as compared to native heparinase I when contacted with the heparin.

22. A method of removing active heparin from a heparin containing fluid comprising:

contacting a heparin containing fluid with the substantially pure polysaccharide lyase as in claims 1, 18, 20 or 21.

23. The method of claim 22 wherein the substantially pure polysaccharide lyase is immobilized on a solid support.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/17310

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N9/88 C08B37/10 A61K38/51 C12N15/70 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 93 08289 A (MASSACHUSETTS INST TECHNOLOGY) 29 April 1993 see the whole document, especially page 4, lines 3-16 and page 19, lines 10-17	1,6-11, 14-17, 22,23 2-5, 18-21
A	---	12
Y	ABSTRACTS OF PAPERS FROM THE FOURTH CHEMICAL CONGRESS OF NORTH AMERICA, vol. 202, no. 1, August 1991, NEW YORK, page a56 XP002026381 LECKBAND D. ET AL.: "Characterization of the active site of heparinase." see abstract	2-5, 18-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 1997	12. 03. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 396 762 A (LANGER ROBERT S ET AL) 2 August 1983 see the whole document, especially col.2, lines 38-47 and examples	13
A	HIGUCHI R.: "Recombinant PCR." 1990 , ACADEMIC PRESS LIMITED , LONDON XP002026386 cited in the application see the whole document	1-12, 14-23
A	ARTERIOSCLEROSIS, vol. 9, no. 1, 1989, pages 21-32, XP000645120 CARDIN A. AND WEINTRAUB H.: "Molecular modeling of protein-glycosaminoglycan interactions." cited in the application see the whole document	6-8
A	CRC CRITICAL REVIEWS IN BIOCHEMISTRY, vol. 8, no. 2, 1980, pages 119-174, XP000645192 KRETSINGER R. ET AL.: "Structure and evolution of calcium-modulated proteins." cited in the application see figure 1	9-11
A	LEWIN B.: "GENES V" 1994 , OXFORD UNIVERSITY PRESS XP002026390 see page 13, last paragraph	12
P,X	BIOCHEMISTRY, vol. 34, 7 November 1995, pages 14441-14448, XP002026383 SASISEKHARAN R. ET AL. : "Heparinase I from Flavobacterium heparinum: The role of the cysteine residue in catalysis as probed by chemical modification and site-directed mutagenesis." see the whole document	1-11, 13-23
P,X	BIOCHEMISTRY, vol. 35, no. 21, 28 May 1996, pages 6846-6852, XP002026384 GODAVARTI R. ET AL.: "Heparinase I from Flavobacterium heparinum. Identification of a critical histidine residue essential for catalysis as probed by chemical modification and site-directed mutagenesis. " see the whole document	1-11, 13-23

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International Application No
PCT/US 96/17310

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J. BIOL. CHEM., vol. 271, no. 6, 9 February 1996, pages 3124-3131, XP002026385 SASISEKHARAN R. ET AL.: "Heparinase I of Flavobacterium heparinum: Mapping and characteization of the heparin binding domain." see the whole document -----	1-11, 13-23

5

INTE: .ATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC1/US 96/17310

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9308289 A	29-04-93	CA 2122004 A	29-04-93
		EP 0610408 A	17-08-94
		JP 7503608 T	20-04-95
US 4396762 A	02-08-83	NONE	